ESR and Monolayer Study of the Localization of Coenzyme Q_{10} in Artificial Membranes

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Abstract. The data obtained from the ESR experiments show a complex, depth dependent effect of CoQ_{10} on the lipid molecules mobility in the bilayer. These effects depend both on its concentration and the temperature. CoQ_{10} disturbs not only the hydrophobic core of the membrane but also the region close to the hydrophilic headgroups of phospholipids. Both these effects could be explained by the fact that the high hydrophobicity of CoQ_{10} causes the molecules to position itself in the interior of the bilayer, but at the same time its water seeking headgroup is located close to the region of the polar headgrops of membrane lipids. The presence of CoQ_{10} in the hydrophobic core has further implications on the properties of membrane intrinsic domain.

Results of monolayer experiments indicate that CoQ_{10} may form aggregates when mixed with PC molecules in the lipid hydrocarbon chain-length dependent manner. CoQ_{10} is not fully miscible with DMPC or DPPC but it is well miscible with the long-chain DSPC molecules. Our suggestion is that CoQ_{10} when present in long-chain phospholipid bilayer, interacts with saturated fatty acyl-chains and adapt the structure which allows such interactions: either parallel to the saturated acyl chains or "pseudo-ring" conformation resembling sterol structure.

Key words: Coenzyme Q_{10} — Electron spin resonance — Monolayer

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Introduction

Coenzyme Q_{10} (CoQ_{10}), which is also known as ubiquinone, is a part of mitochondrial respiratory chain. But mitochondria are not the only site of CoQ_{10} in a cell. There are strong evidence that CoQ_{10} is also present in the plasma membrane, endoplasmic reticulum, lysosome, Golgi apparatus and nuclear membranes (Shapiro and Saliou 2001). Ubiquinol (the reduced form of CoQ_{10}) has very potent antioxidant functions, very similar to those of vitamin E. It is believed that CoQ_{10} prevents not only lipid peroxidation by reducing peroxyl radicals but also regenerates endogenous vitamin E levels in the membranes (Gilgun-Sherki et al. 2001). Though many different experiments have been performed (Spinsi et al. 1978; Quinn and Esfahani 1980; Ulrich et al. 1985; Fato et al. 1986; Jemioła-Rzemińska et al. 1996, 2001; Skowronek et al. 1996) to explain the possible localization of the CoQ_{10} in mitochondrial membranes, the detailed picture is still unclear. The location of the shorter ubiquinones in the membrane is much easier to define: Q_2 localizes close to the membrane surface and does not penetrate the interior regions (Skowronek et al. 1996); Q_4 lines perpendicular to the membrane plane with its tail reaching the internal part of the membrane (Skowronek et al. 1996). A fully extended CoQ_{10} molecule is $\sim$50 Å in length, which is too long to be accommodated perpendicularly to the membrane plane (Di Bernardo et al. 1998). The fast rate of flip-flop, observed for those molecules, indicates different localization of the CoQ_{10} in the membrane (Jemioła-Rzemińska et al. 1996; Di Bernardo et al. 1998). Q_{10} is thought not only to locate itself in the midplane of the membrane (Ulrich et al. 1985; Di Bernardo et al. 1998) but also to form CoQ_{10} rich pools inside the bilayer (Jemioła-Rzemińska et al. 1996, Ulrich et al. 1985). The presence of CoQ_{10} in the interior of the membrane enables the ubiquinone to move in two dimensions quickly (Fato et al. 1986) but it does not explain how the quinone traverse from one bilayer leaflet to the other. The most probable location of CoQ_{10} is the middle of the bilayer with the quinone ring oscillating between the bilayer surfaces (Fato et al. 1986) by bending the long tail at the third isoprenoid unit (Samori et al. 1992). The aim of the experiments reported in the paper was to test the hypothesis concerning the location of CoQ_{10} in lipid bilayer as well as to establish its miscibility with phosphatidylcholine (PC) of various hydrocarbon chain length.

Materials and Methods

Chemicals

The DMPC (dimyristoylphosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine) and DSPC (distearoylphsphatidylcholine) were purchased from Sigma, Egg PC (phosphatidylcholine) was obtained from Lipid Products, CoQ_{10} was from Jemo-pharm A/S. Phospahate-buffered saline (PBS) buffer (10 mmol/l phosphate buffer, 0.27 mmol/l KCl, 13.7 mmol/l NaCl, pH 7.4) was used for the liposome

preparation. The stearic acid spin labels: 5-doksyl-stearic acid (C5-DSA) and 16-doksyl-stearic acid (C16-DSA) were from Aldrich.

Multilamellar vesicles preparation

Multilamellar vesicles for the electron spin resonance (ESR) studies were prepared as described previously (Kozubek et al. 1988) by mixing chloroform solutions of appropriate compounds, evaporation of the solvent under stream of nitrogen and then under vacuum for at least 12 h in the dark. The dried film was hydrated with PBS. The final suspension contained 1mg of lipids *per* milliliter of suspension. The molar ratio of the lipids was DMPC : DPPC : DSPC (62 : 29 : 9), the vesicles contained 0, 5, 10 and 20% of CoQ_{10} (w/w). The spin label probe concentration in the sample was 0.25 μmol/l for C5-DSA (0.018 mol%) and 0.4 μmol/l for C16-DSA (0.029 mol%).

ESR studies

Aliquots of liposome suspensions were put into capillaries and electron spin resonance spectra were recorded in a Brucker spectrometer with a temperature control device.

The order parameter S, which is a measure of the average angular deviation of the fatty acid acyl chain of the spin label probe at the nitroxide group from the average orientation of the fatty acids in the membrane (Hubbel and McConnell 1971), reflects the membrane fluidity state. The S values were obtained from the C5-DSA spectra using the equation (Sefton and Gaffney 1974)

$$S = \frac{T'_{\parallel} - (T'_{\perp} + C)}{T'_{\parallel} - 2(T'_{\perp} + C)} \cdot 1.723$$

where $T'_{\parallel}$ and $T'_{\perp}$ were calculated from the ESR spectra and C is a correction for the deviation of the observed value of $T'_{\perp}$ from the actual state:

$$C = 1.4 - 0.053(T'_{\parallel} - T'_{\perp})\mathrm{G}$$

The S value varies from 1 to 0, where 1 is characteristic for completely rigid lipid environment and the reduction in these values means the increase in the mobility of the components of the membrane. For the C16-DSA labeled liposomes, rotational correlation time τ was calculated using equation (Ligeti and Horváth 1980)

$$\tau = K(\sqrt{h_0/h_{-1}} - 1)W_0$$

where W_0 and h_0 are the width and height of the central field line of the spectrum, respectively, and h_{-1} is the height in Gauss (G) of the high field line. The decrease in the value of rotational correlation time τ means increased rotational motion of the label.

ESR spectra simulation

The lipid bilayer can be an assemblage of different domain populations, which is a consequence of distinct molecular composition (Štrancar et al. 2000; Arsov et. al 2002). In each such domain, spin probes would be present. Assuming the existence of lateral domains in the membrane, each observed ESR spectrum should be a superimposition of the spectral components that identify the membrane heterogeneity in the sample (Arsov et. al 2002). The spectra were decomposited with the help of EPRSIM software version 4.2 (© Janez Štrancar, downloaded from: http://www2.ijs.si/∼jstrancar/software.htm). The spectra were decomposited as follows: each spectrum was first decomposited for three different domain populations and the parameters were optimized with the Simplex algorithm. The goodness of fit was evaluated with the χ_1^2 objective function which is the sum of squared residuals between the experimental and the simulated spectra divided by the squared standard deviation of the experimental points and by the number of points in the experimental spectrum (in our case the number of points $n = 1024$). The objective function is supposed to be the best choice when describing membrane spectra because it sums residuals in "islands", spanned between the two neighboring crossings of the experimental and simulated spectra, weighted by a power of the individual island lengths (Štrancar et al. 2000).

After the first step, the p_g and p_A correction factors were blocked and the fit was further optimized with the Genetic optimization. The next step was to increase the number of domains by one and repeating the whole procedure. To establish the most probable number of different domains in a spectrum we compared the χ_1^2 value of each spectra simulated with 3, 4 and 5 different domains. The lowest value of the χ_1^2 function was the measure of the best fit, and therefore taken as the most probable number of different domains existing in the membrane.

Monolayer preparation and data analysis

Chloroform solutions of CoQ_{10} or its (5–40 % w/w) mixtures with PC were prepared at lipid concentration of 1 mg/ml. As the subphase, deionized water was used (pH 7.0). The isotherms were recorded using a 70 cm^2 teflon Langmuir trough fitted with motorized compression barrier (Nima Technology) equipped with a pressure sensor and Wilhelmy plate. The barrier speed was set to 2 cm^2/min. The trough was surrounded by water jacket that provided a temperature control (22.5 ℃) and was placed in a chamber facilitating flushing with a nitrogen stream (to avoid oxidizing the CoQ_{10}). For each combination of CoQ_{10} – lipid monolayer, 3–6 isotherms were recorded and surface pressure Π *vs.* molecular area A were plotted.

To establish the miscibility of the components in the monolayer, the excess free energy of mixing ΔG_m^{ex} was calculated. The parameter may give some details about the thermodynamics of the system and may provide further information about the energetics of the miscibility process and possible specific interactions between the two components. The positive values of ΔG_m^{ex} mean that the mutual interactions between the two different components are weaker than interactions

between the pure component molecules themselves (at least one component forms bidimensional aggregates). If $\Delta G_{\mathrm{m}}^{\mathrm{ex}} = 0$ than the mixing is ideal.

$\Delta G_{\mathrm{m}}^{\mathrm{ex}}$ was calculated according to Maget-Dana (1999), i.e.

$$\Delta G_{\mathrm{m}}^{\mathrm{ex}} = \int_{\Pi^0}^{\Pi} A_{12}\mathrm{d}\Pi - X_1 \int_{\Pi^0}^{\Pi} A_1\mathrm{d}\Pi - X_2 \int_{\Pi^0}^{\Pi} A_2\mathrm{d}\Pi$$

where Π^0 was 0 mN/m and Π was 10 mN/m. The area of each isotherm was calculated using SigmaPlot 8.0.

Results

ESR

CoQ_{10} is a highly hydrophobic compound, which solubilizes well in hydrophobic solvents, so its midplane localisation in the lipid bilayer was postulated (Spinsi et al. 1978; Quinn and Esfahani 1980; Ulrich et al. 1985; Jemioła-Rzemińska et al. 1996, 2001; Skowronek et al. 1996).

CoQ_{10}-induced changes in membrane properties were measured by spin labeling with fatty acid analogues in 5C and 16C regions. When 5C-DSA was used for labeling at 20 ℃, substantial decrease in order parameter S for 5 % CoQ_{10} was observed (Fig. 1). The presence of higher concentration of CoQ_{10} in the membrane at the same temperature induced an increase in S to the values close to the control membranes. At temperatures higher than 25 ℃, only an increase in order parameter S induced by the presence of CoQ_{10} (rather independent of concentration) was observed (Fig. 1)

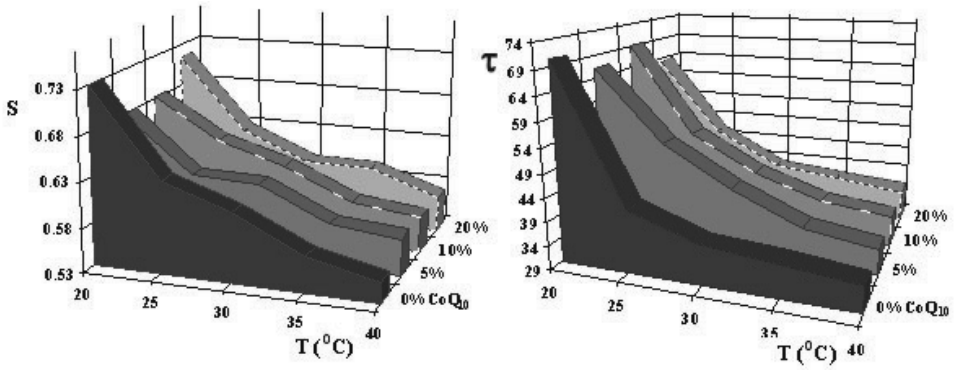

Figure 1. The effect of various concentrations of Coenzyme Q_{10} (CoQ_{10}) on the order parameter S for C5-DSA labeled liposomes (left panel) and on the rotational correlation time τ (in ns) of liposomes labeled with C16-DSA (right panel). Liposomes were composed from DMPC : DPPC : DSPC (62 : 29 : 9) and contained various concentrations of CoQ_{10}.

Rotational correlation time τ at 20 ℃ was slightly lower for samples containing CoQ_{10} but the changes were not essential (∼10 %). At temperatures 25 ℃ and higher, a concentration dependent increase in τ values was observed (Fig. 1). The largest increase (∼34 %) was observed at 25 ℃ for 20 % CoQ_{10} in the liposomal membrane. This could implicate microheterogeneity of the membrane hydrophobic phase.

ESR spectra simulation

Because of the quite complex structure of our liposomes, there are several possibilities for interactions between the different membrane components. The presence of lateral domains i.e. spatially limited regions in the membrane which have at least one measurable properties that distinguish from neighboring regions (Bloom and Thewal 1995), cannot be neglected. Decomposition of the spectra according to the method of Štrancar et al. (2000) of liposomes labeled with C5-DSA showed that the best fit was achieved with four different signals. For the control spectra as well as for the samples with CoQ_{10}, two of them were anisotropic and two were isotropic independent on the temperature. The comparison of χ_1^2 values of spectra showed that the best fit is always achieved with four domains and that it is correlated neither with the temperature nor the presence of CoQ_{10} in the membrane (data not shown).

On the other hand, the analysis of the spectra for the samples with C16-DSA revealed differences in the number of possible domains. Membranes which

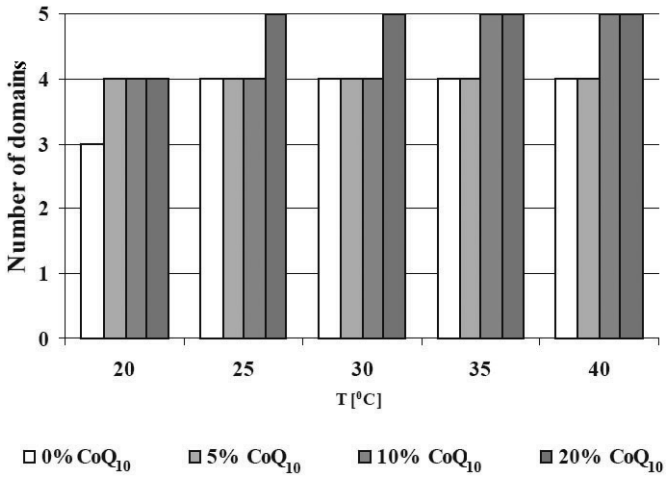

Figure 2. The number of domains in the optimized ESR spectra obtained with the C16-DSA established after the comparison of the χ_1^2 value of each spectra simulated with 3, 4 and 5 different domains. The lowest value of the χ_1^2 function was the measure of the best fit, and therefore taken as the most probable number of different domains existing in the membrane. (The lowest values of χ_1^2 of the simulated spectra are shown in Table 1).

Table 1. The lowest values of χ_1^2 of the simulated spectra. (The number of domains for which the simulation recorded the lowest value of χ_1^2 are presented in Fig. 2)

Spin label	CoQ_{10} (%)	20 °C	25 °C	30 °C	35 °C	40 °C
C5-DSA	0	2.74	3.41	3.87	4.43	3.33
	5	5.63	8.15	5.38	12.44	12.34
	10	5.13	20.10	21.02	13.07	11.02
	20	3.29	9.50	9.03	10.14	7.32
C16-DSA	0	8.28	5.66	7.95	7.35	11.69
	5	4.83	10.24	9.31	6.30	9.02
	10	5.22	4.25	14.19	9.42	9.38
	20	3.16	2.78	6.20	7.29	8.83

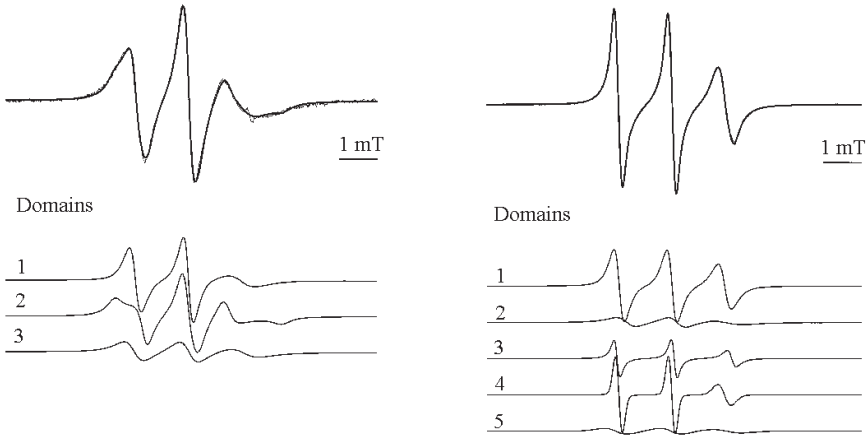

Figure 3. Examples of the simulation of spectra obtained for control liposomes (DMPC : DPPC : DSPC) at temperature 20 °C (left panel) and for vesicles containing 20 % CoQ_{10} at temperature 40 °C (right panel).

did not contain CoQ_{10} revealed presence of 3 domains at 20 °C and 4 at higher temperatures, while in membranes enriched in $CoQ_{10,}$ 4 domains at 20 °C and up to 5 domains at higher temperatures for 10 and 20 % CoQ_{10} were detected (Fig. 2 and Table 1). Examples of the decomposited spectra for control and CoQ_{10} containing membranes are shown in Fig. 3.

The above results seem to confirm the conclusion from the calculations of S and τ values (Fig. 1) and indicate much larger effect of CoQ_{10} on the properties of the interior of the membrane bilayer, although small effect on the order parameter S measured using C5-DSA could also be seen.

Monolayer

The next set of experiments is an answer to the question how the presence of CoQ_{10} in monolayers composed of various fatty acyl chain length PCs changes the properties of these monolayers.

The isotherms collected in experiments which are presented in Figs. 4–6 were repeated 3–6 times and were fully recurrent for each of the mixtures used. In the monolayer experiments, a mixture of CoQ_{10} (ubiquinone) and $CoQ_{10}H_2$ (ubiquinol) was used. Such a mixture forms a stable monolayer and has a collapse pressure that lies between those for pure CoQ_{10} and $CoQ_{10}H_2$ (Fig. 4) (Quinn and Esfahani 1980). CoQ_{10} when mixed with phospholipids forms a stable monolayer as well. Fig. 4 represents results recorded for mixed DMPC-CoQ_{10} mono-

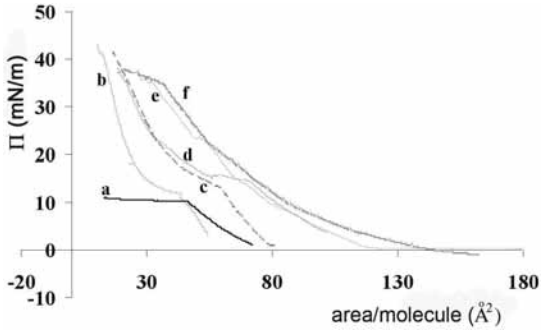

Figure 4. Surface pressure-area *per* molecule (Π-A) isotherms of DMPC-CoQ_{10} mixed monolayers: (a) CoQ_{10}, (b) DMPC : CoQ_{10} (60 : 40), (c) DMPC : CoQ_{10} (80 : 20), (d) DMPC : CoQ_{10} (90 : 10), (e) DMPC : CoQ_{10} (95 : 5), (f) DMPC.

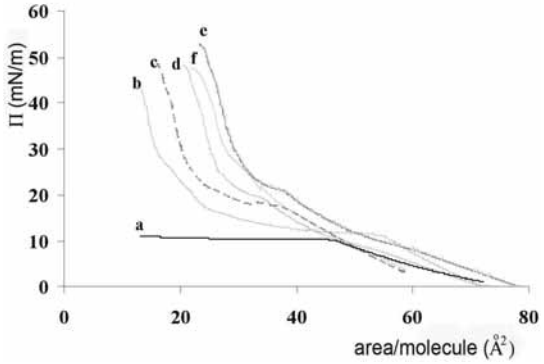

Figure 5. Surface pressure-area *per* molecule (Π-A) isotherms of DPPC-CoQ_{10} mixed monolayers: (a) CoQ_{10}, (b) DPPC : CoQ_{10} (60 : 40), (c) DPPC : CoQ_{10} (80 : 20), (d) DPPC : CoQ_{10} (90 : 10), (e) DPPC : CoQ_{10} (95 : 5), (f) DPPC.

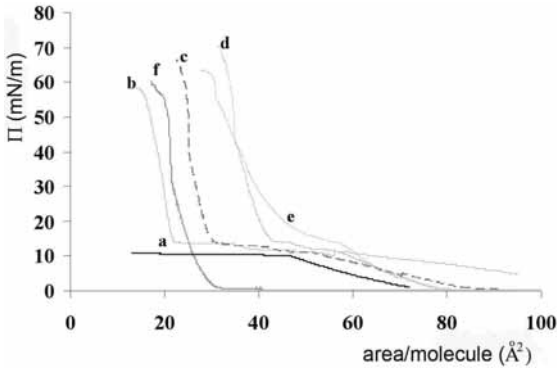

Figure 6. Surface pressure-area *per* molecule (Π-A) isotherms of DSPC-CoQ_{10} mixed monolayers: (a) CoQ_{10}, (b) DSPC : CoQ_{10} (60 : 40), (c) DSPC : CoQ_{10} (80 : 20), (d) DSPC : CoQ_{10} (90 : 10), (e) DSPC : CoQ_{10} (95 : 5), (f) DSPC.

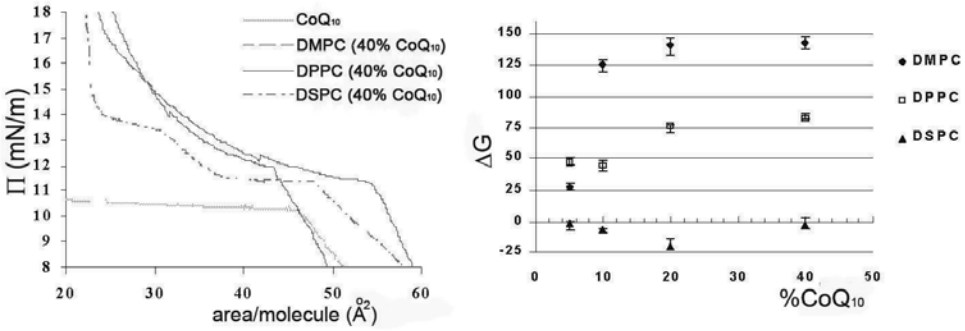

Figure 7. A two step squeezing out action for DSPC-CoQ_{10} monolayers in comparison with shorter-chain phosphatidylcholines (PCs; left panel): a) CoQ_{10}, b) DMPC-CoQ_{10} (60 : 40) monolayer, c) DPPC-CoQ_{10} (60 : 40), d) DSPC-CoQ_{10} (60 : 40). The difference between one step plateau in DMPC or DPPC isotherms and the two "kinks" in DSPC-CoQ_{10} monolayer might be caused by different miscibility of used PCs with Q_{10}. In the right panel the dependence of $\Delta G_{\mathrm{m}}^{\mathrm{ex}}$ values on CoQ_{10} content for its mixtures with different PCs (indicated in the legend) is shown.

layers and they are very similar to the results obtained by Quinn and Esfahani (1980).

The results recorded for DPPC-CoQ_{10} monolayers resemble those obtained for DMPC-CoQ_{10} mixtures. In both cases each isotherm contains a characteristic "kink" (Fig. 5). With the increase in CoQ_{10} content in the mixture, the kink was deeper and more "sharp". However, the isotherms recorded for DSPC-CoQ_{10} mixtures show not one but two plateau regions (Fig. 7, left panel). It has been

postulated (Quinn and Esfahani 1980) that at a given pressure, CoQ_{10} molecules are being pushed out of the monolayer. This effect would explain the behavior of the DMPC- and DPPC-CoQ_{10} mixtures but in the case of DSPC-CoQ_{10} this explanation turns out to be not satisfactory. In order to get some more information about the miscibility of both components in each mixture, a quantitative analysis of isotherms, namely $\Delta G_{\mathrm{m}}^{\mathrm{ex}}$ calculations were performed (Fig. 7, right panel). As it is shown in this figure, the $\Delta G_{\mathrm{m}}^{\mathrm{ex}}$ values obtained for DSPC monolayer are substantially lower than, those obtained for DMPC and DPPC monolayers, and moreover all of them are negative. Therefore we can anticipate that the interactions of CoQ_{10} with long fatty acyl chains of DSPC can occur.

Discussion

The results presented here demonstrate that although CoQ_{10} molecule is highly hydrophobic, its localization in the membrane is not only restricted to the membrane interior, however, its presence does not implicate the formation of additional lateral domain(s) in the outer part of the bilayer. Data published previously by others show that CoQ_{10} resides mainly in the middle of bilayer's hydrophobic part (Ulrich et al. 1985; Fato et al. 1986; Di Bernardo et al. 1998) but also that at least some part of the CoQ_{10} causes perturbations in the outer parts (Jemioła-Rzemińska et al. 1996). Our data indicate that CoQ_{10} strongly affects the hydrophobic core of the membrane. It is well visible when comparing the τ values, but the effect is remarkable also after the decomposition of the spectra. At 20 ℃, CoQ_{10} containing liposomes seem to have one more domain in comparison to the control sample. The fourth domain may be discerned from the control spectra at the temperature of 25 ℃ and above. But at 25 ℃, the sample containing 20 % CoQ_{10} seems to have five different domains. Also the 10 % CoQ_{10} liposomes display the existence of five domains but only at 35 ℃ and above. The raise of the temperature implies generally the formation of additional domains in the decomposited spectra obtained with C16-DSA. Also the increase in CoQ_{10} influences the appearance of additional domain (Fig. 2). To act as an electron transporter and a proton carrier, CoQ_{10} must be able to interact with the water-lipid interfaces in the bilayer. The results of the experiments presented here shows that ubiquinone's miscibility depends on the fatty acyl chain length of the PC's lipids. Jemioła-Rzemińska et al. (1996) showed that if CoQ_{10} concentration in the membrane exceeds 2 mol %, it forms pool inside the bilayer. It is demonstrated here that the presence of long-chain PC moves this threshold towards the higher values of CoQ_{10} concentrations. The data show that CoQ_{10} does not mix well with DMPC or DPPC but its miscibility rises with the increase in length of fatty acyl chains of lipid. The CoQ_{10} is better miscible with the DPPC than DMPC and is much better miscible with the DSPC molecules. We conclude therefore that, in DMPC and DPPC monolayers, CoQ_{10} molecules are probably organized in some kind of clusters (probably with their tails interacting together laying over the shorter tails of the PC molecules). The observed kink is,

as has been shown before (Quinn and Esfahani 1980), a result of squeezing out the CoQ_{10} molecules from the monolayer. In the case of DSPC-CoQ_{10} mixtures, the isotherm indicates that the two compounds mix well despite the fact that the CoQ_{10} molecule is at least twice as long as the DSPC molecule. For the long tail of CoQ_{10} being accommodated within the DSPC fatty acyl chains, more complicated structure may be proposed. With increasing pressure, however, the long tails of CoQ_{10} are likely to be pushed out what results in its clustering (first kink) and than, at the higher pressures, the molecule is pushed out of the monolayer altogether. The presence of DSPC in the bilayer may cause a formation of domains where CoQ_{10} molecules are located completely parallel to the fatty acyl chains in the membrane or, more probably, it can adapt folded, "pseudo-ring, squalene-like" conformation resembling sterol structure. These structures are probably stabilised by the interactions of CoQ_{10} with long fatty acyl chains of DSPC what is suggested by negative values of ΔG obtained for DSPC-CoQ_{10} monolayers.

The results presented here show that the location of hydrophobic CoQ_{10} is not restricted to the midplane of the membrane. The interference with lipid organization in bilayers and the shape of monolayer isotherms indicate that: firstly, the CoQ_{10} also affects the region close to the polar headgroup region and secondly, when at high concentrations it forms clusters in both bilayers and monolayers. Long fatty acyl chain phospholipid are much better miscible with CoQ_{10} perhaps due to the interactions of CoQ_{10} with saturated fatty acyl chains. Such molecule localization within the membrane satisfies the functional requirement when CoQ_{10} must have an access to the polar headgroups of the membrane.

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